CONTRIBUTIONS TO THE MICROBIOLOGY OF AUSTRALIAN SOILS. II.

A COMPARISON OF THE ROSSI-CHOLODNY METHOD AND THE PLATE METHOD FOR STUDYING THE SOIL MICROFLORA.

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(Plate vi.)

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Introduction.

Direct microscopical methods for the observation and enumeration of soil microorganisms have received considerable attention during recent years. A useful technique for this purpose was first developed by Conn (1918) and later modified by Winogradsky (1925). These methods have shown that soils contain bacteria in numbers many times as high as those obtained by the current plate or dilution methods. The figures, however, are often very erratic, as shown by Kühlmorgen-Hille (1928), and the same would presumably apply to the essentially similar methods of Vande Velde and Verbelen (1930) and Fehér (1932). More reliable results may be obtained by means of an improved technique devised by Gray and Thornton (1928). Another method, specially adapted to the study of soil protozoa, has been developed by Koffmann (1928-31). A common feature of these methods is that the microorganisms are made visible by staining of a soil suspension, either in wet mounts, as in one of the modifications of the method of Koffmann, or else in dried films. Although adequate for the study of bacteria and protozoa, such methods are less suitable for the actinomycetes and fungi, which organisms consist of mycelia that may be broken up and scattered by the preparation of the soil suspension (this is also one of the main disadvantages of the usual plate methods); indeed, fungus mycelium may be altogether absent from the microscopical picture (Conn, 1918). The first attempt to find an adequate method for the microscopical study of fungi and actinomycetes in soil was made by Conn (1922), but this method does not seem to have been widely used, and may not always lead to reliable results (McLennan, 1928). A more promising way of attacking the problem appears to have been found by Cholodny (1930) and Rossi (1928, cit. after Cholodny) who, independently of each other, devised a simple method consisting in placing microscopic slides in the undisturbed soil for a certain interval of time, and fixing and staining the microorganisms growing upon and adhering to them. Conn (1932) has shown how this method may be applied to laboratory experiments with soil. Beautiful pictures of the soil microflora and -fauna, obtained by the same method, have been published by Demeter and Mossel (1933), who also discuss the limitations of the method, particularly the facts that it is only very roughly quantitative,* and that, like

* In view of the irregular distribution of the organisms and the fact that the coarser soil particles are washed off the slides, it would appear highly doubtful whether the combined counting and weighing of the slides, as attempted by Verplancke (1932), could give reliable quantitative results.

the other microscopical methods, it does not supply any cultures of the organisms observed. It must also be realized that when the slides are left in the soil for periods of several weeks, we do not obtain a picture of the soil population at any given moment (such as found by the other microscopical methods and the culture methods), but one resulting from a shorter or longer process of growth of organisms on the slides. On the other hand, the Rossi-Cholodny method shows the fungi and the actinomycetes in their actual, undisturbed state and, like the other microscopical methods, it reveals the presence of organisms not amenable to study by plate or dilution methods. It would, therefore, seem very interesting to compare the results obtained by means of these two methods; an attempt hereat is presented in this contribution.

Methods.

Various kinds of organic matter were added to soils in the laboratory, and the resulting changes in the microflora were followed both by the plate method and by the Rossi-Cholodny method as recommended by Conn (1932). Two different soils were used: one (*A*) a loam, rich in organic matter and lime, of pH 7·3, from a flower bed, the other (*B*) a heavy loam, very rich in organic matter, of pH 5·4, from uncultivated grass land. The samples had been air-dried, ground and sieved before use. Portions of about 500 gm. of moist soil (moisture adjusted to approximately 70% of the water-holding capacity of each soil) were placed in big Petri dishes (internal diameter 18·5 cm., depth 4·5 cm.), forming a layer about 3 cm. deep, and a number of microscopic slides were placed vertically in the soil, with their long sides against the bottom of the dish. Previous to use, the slides had been carefully cleaned by boiling with potassium bichromate and sulphuric acid, washing with water, and storing in alcohol which was removed by flaming. After various periods of time, one or two slides were taken out for microscopical examination, and at the same time samples (8-10 gm.) of the surrounding soil were taken out for plate counts and moisture determinations. The dishes with soil were kept at room temperature (21–26°C.), and only small changes in the moisture content took place during the course of the experiments. The following series of experiments were run:

A. (Alkaline garden soil.)

First series: *a.*—Control soil (no addition); *b.*—Soil + 1·0% mannite; *c.*—Soil + 0·5% dry milk; *d.*—Soil + 0·5% keratin.

Second series: *a.*—Control soil; *b.*—Soil + 1·0% cellulose + 0·05% $NaNO_3$; *c.*—Soil + 1·0% liquid paraffine + 0·05% $NaNO_3$.

B. (Acid grass soil.)

First series: *a.*—Control soil; *b.*—Soil + 1·0% cellulose + 0·1% $(NH_4)_2SO_4$.

Second series: *a.*—Control soil; *b.*—Soil + 1·0% mannite; *c.*—Soil + 0·5% dry milk.

Besides these, some experiments were carried out on various soils without addition of organic matter. Since no large quantities of these soils were available, they were kept in smaller glass containers as used by Conn (1932). The media and the technique of plate counting were the same as used in a previous series of experiments (Jensen, 1934), except that the plates were incubated at room temperature. The mannite-asparagine-agar devised by Thornton (1922) was also used for counting bacteria and actinomycetes in the alkaline soil with mannite. In the first series of experiments with the acid soil, as well as in some of the soils without additions, counts of fungus spores were made according to

the method of McLennan (1928): drying of the soil *in vacuo* for 3 days, which kills the vegetative mycelia while leaving the spores intact. A modification was introduced in the staining of the preparations for microscopical examination, instead of simple staining with Rose bengale (Conn, 1918) or erythrosine (Winogradsky, 1925). Preliminary experiments showed that a differentiation of the soil microflora could be obtained by staining according to the Gram method; the soil colloids are decolourized by the alcohol treatment, and are not re-stained when erythrosine or Rose bengale* is used as a counterstain for the Gram-negative soil organisms. Barthel (1918, 1919) has previously shown that good pictures of Gram-positive organisms may be obtained by Gram-staining of smears from cultures in sterilized soil. Also Demeter and Mossel (1933), whose paper appeared while the present work was in progress, suggest Gram-staining, but apparently without yet having used it for soil. In my experiments the following technique has been used:

1. After air-drying and removal of sand-grains, the slide is passed through a Bunsen flame to fix the microorganisms.—2. Staining 2–3 minutes with Crystal-violet-ammonium oxalate-solution (Gram-Hucker).—After washing, treatment for 1–2 minutes with Lugol's iodine.—3. Washing, drying, and differentiation 4–5 minutes with absolute alcohol which is renewed 3–4 times.—4. Drying, and counter-staining 10–12 minutes on water bath at 60–70°C. with Rose bengale solution after Conn (1932).

In some cases a search was made for acid-fast organisms by staining with hot carbol-fuchsin and differentiation 30–60 seconds with 5% sulphuric acid.

Experimental Results.

The results of the plate counts in the first series of experiments with soil A are shown in Table 1.

TABLE 1.[1]

Numbers of Bacteria and Actinomycetes in Alkaline Garden Soil. Series I.

Time.	Control.		Mannite.				Milk.		Keratin.	
			Casein Agar.		Mann. Agar.					
	Bact.	Act.	Bact.	Act.	Bact.	Act.	Bact.	Act.	Bact.	Act.
Start ..	50·9	3·2	50·9	3·2	—	—	50·9	3·2	50·9	3·2
3 days	44·4	4·5	112·9	1·6	—	—	1529·0	45·5	—	—
7 ,,	26·7	4·5	412·9	2·0	423·8	4·3	489·0	17·9	206·5	25·6
11 ,,	25·1	4·1	—	—	294·9	1·9	408·7	26·9	196·5	31·6
16 ,,	31·4	4·3	301·9	3·1	350·0	8·0	342·9	23·7	265·4	20·2
22 ,,	18·2	3·5	253·3	2·3	238·7	3·1	377·5	18·3	208·3	17·9

[1] In this, as well as the following tables, bacteria and actinomycetes are expressed as millions, and fungi as thousands per gm. of dry soil.

* Of the batches of dyes at my disposal, Rose bengale gave rather better results than erythrosine.

The examination of the slides gave the following results:

a. Control soil.—The majority of the bacteria in this soil is represented by small Gram-negative rods, occurring singly, in short chains, or in colonies of greatly varying size. The rest of the bacterial flora is made up of (1) small short Gram-positive rods, mostly in pairs or small clusters, showing the characteristic angular arrangement of the corynebacteria, (2) big Gram-negative cocci, 1·0–1·4μ, (3) long, unbranched, Gram-negative filaments, 0·8–1·0μ thick, often, especially after 16–22 days, occurring in characteristic spools, very much resembling the Fig. 11*a* of Demeter and Mossel (1933), an object which these authors interpret as an actinomyces. Vegetative mycelia of actinomycetes are generally scarce, often Gram-negative; a strong development in isolated patches was seen after 11 days. Chains of Gram-positive actinomyces-spores are constantly seen, although not in very large numbers; a single, very extensive system of mycelium producing aerial spores was seen after 16 days. Fungus mycelium was constantly present, but not very abundant; the hyphae frequently appeared decayed and badly staining, with Gram-negative rods or long Gram-negative filaments clinging to them (cf. Conn, 1932, and Demeter and Mossel, 1933). Fungus spores could not be observed with certainty.

b. Soil + Mannite.—This soil showed throughout the whole course of the experiment a strong development of typical *Azotobacter*-organisms: big oval to spherical cells, 2·0–3·0 × 2·5–4·5μ, mostly in big, scattered colonies, with markedly granular cytoplasm, nearly all Gram-negative, some with Gram-positive granules, a few partially or almost wholly Gram-positive (Pl. vi, fig. 8). This strong multiplication of *Azotobacter* is clearly reflected in the counts on the agar plates, where a great rise in the numbers of colonies sets in, reaches its maximum by the 7th day, and then recedes somewhat. This increase is for the very largest part due to *Az. chroococcum*, whose colonies account for some 80–90% of the total numbers of colonies on both media, which do not differ significantly from each other. Petersen (1925) has previously shown that *Az. chroococcum* will develop on dextrose-casein-agar by direct plating from soil with addition of mannite. Besides *Azotobacter*, there was, during the whole experiment, a number of small Gram-positive rods, sometimes branched, resembling corynebacteria (Pl. vi, fig. 11). The small Gram-negative rods and the long Gram-negative filaments were rare, especially during the first 3–11 days. Actinomyces-mycelia (largely Gram-negative) and -spores were present, but not more conspicuous than in the control soil; in agreement herewith, we observe no very considerable changes in the numbers of actinomyces-colonies on the agar plates. The fungus flora did not seem to undergo any distinct change as compared with the control soil, except that a number of spindle-shaped fungal spores (*Fusarium*?) were seen after 16 days.

c. Soil + Milk.—The slides from this soil showed, after 3 days, a huge development of small, Gram-negative, rod-shaped bacteria, which from the 7th day to the end of the experiment appeared somewhat less abundant. The other types of bacteria (small Gram-positive rods, big Gram-negative cocci, and long Gram-negative filaments) were very scarce after 3 days, later somewhat more prominent, although still forming only a small fraction of the flora. Contrary to expectation, no vegetative cells of spore-forming bacteria were observed, although some bacterial spores were seen after 3 days. The actinomycetes showed a most impressive development; their vegetative mycelia were remarkably

scarce, often Gram-negative, but throughout the whole experiment there was an abundance of Gram-positive actinomyces-spores, mostly 1·0 × 1·2–1·6μ, in fine, long, fairly straight chains (Pl. vi, fig. 5), with some tendency to break up and scatter after 16–22 days. It was very striking that these spore-chains were nearly always situated in those regions of the slides that were free from soil colloids. The counts on agar plates (Table 1) show a rapid increase and a slow decrease in bacteria (small rods) and actinomycetes, like the microscopical examination. The fungus mycelia did not show any considerable development on the slides, although some hyphae of *Mucor*-type were seen. From the 7th day to the end of the experiment, a considerable number of amoebae were seen; they were generally 12–20μ in diameter, Gram-negative, and contained numerous Gram-positive actinomyces-spores (Pl. vi, fig. 7).*

d. Soil + Keratin.—There was here, after 3 days, a considerable increase in small Gram-negative rods, although not so impressive as in the soil with milk. Later, small and middle-sized Gram-positive rods resembling corynebacteria (Pl. vi, fig. 10) became quite numerous, together with the big Gram-negative cocci and the long Gram-negative filaments. The actinomycetes developed strongly after 3 and 7 days, showing vegetative mycelia (Pl. vi, fig. 4) as well as chains of spores (Pl. vi, fig. 6), the latter sometimes forming spirals. The vegetative mycelia became less conspicuous after 11 days, but the spores remained abundant all through the experiment. Fungal hyphae of a *Mucor*-type were quite numerous after 3–7 days, later disappearing. In this soil, too, a considerable number of amoebae containing actinomyces-spores were observed. The figures in Table 1 show, corresponding to the microscopical picture, an increase in bacteria as well as actinomycetes, but less than in the soil with milk, in agreement with the less abundant development of organisms on the slides. A large number of corynebacteria appeared on the plates.

Table 2 shows the results of the plate counts from the second series of experiments with this soil.

TABLE 2.

Numbers of Bacteria and Actinomycetes in Alkaline Garden Soil. Series II.

Time.	Control.		Cellulose.		Paraffine.	
	Bact.	Act.	Bact.	Act.	Bact.	Act.
Start	37·8	5·3	37·8	5·3	37·8	5·3
5 days	43·1	5·5	53·1	6·3	68·0	12·6
10 „	50·4	6·2	101·4	5·0	65·9	13·1
15 „	36·7	4·4	52·5	3·6	47·2	14·6

The microscopical examination gave the following results:

a. Control soil.—The microscopical picture was in no way different from that of the first series. Staining for acid-fast organisms showed that small rods of this character were present, but very rare.

* Severtzova (1928) found amoebae unable to feed on actinomycetes in agar culture, where they could probably only get into contact with the vegetative mycelium, but no information seems available as to the power of amoebae to ingest the aerial spores of actinomycetes.

b. Soil + Cellulose.—The bacterial flora in this soil underwent little visible change after 5 days; after 10 and 15 days there was a notable development of big oval Gram-negative organisms, 1·0 × 1·5–2·0μ, mostly in clumps around the fibres of cellulose, and of some quite small, thin, curved threads resembling the cellulose-decomposing *Spirochaeta cytophaga.* Numerous small Gram-positive rods were also seen, and the long Gram-negative filaments produced very fine "spools" in this soil (Pl. vi, fig. 9). Fungus mycelia appeared in considerable amounts after 15 days, but the actinomycetes showed no change in comparison with the control soil. In agreement herewith, the numbers of actinomycetes remained virtually unchanged in the plate counts, and the bacteria showed only a moderate increase by the 10th day. An attempt was made to determine the approximate number of cellulose-decomposing bacteria in this and the control soil by the dilution method: inoculation from increasing dilutions of soil suspension in test-tubes with a strip of filter paper and a mineral nutrient solution containing $NaNO_3$ as a source of nitrogen. *Spirochaeta cytophaga* developed from the soil with cellulose in dilutions up to 1:4,000, but from the control soil only in the dilution of 1:10.

Soil + Paraffine.—The slides from this soil showed some development of short, plump, almost coccoid, Gram-negative or Gram-variable rods in large clusters. Small Gram-positive rods were also quite numerous. There was a considerable growth of vegetative actinomyces-mycelia, some of them richly branching, Gram-negative, with marked belt-staining, others chiefly Gram-positive, divided into segments of irregular size and shape, suggesting a *Proactinomyces*-type. Very few actinomyces-spores and fungus mycelia were seen. Staining for acid-fast organisms revealed, after 10 and 15 days, a small number of minute acid-fast rods, mostly in angular or parallel clusters (Pl. vi, fig. 13) and clumps of small acid-fast cocci. The plate counts show only a small increase in bacteria, but a quite definite multiplication of actinomyces-colonies. About 25% of these were represented by pink colonies of a partially acid-fast organism which, after isolation, proved to be a form closely resembling *Proact. polychromogenes*, an organism that has previously been found multiplying in soil with addition of paraffine (Jensen, 1931). This may have been identical with the acid-fast organisms observed on the slides.

The results of the plate counts from the acid soil B are shown in Table 3.

The first series of experiments gave the following results:

a. Control Soil.—This was quite rich in bacteria, mostly small, nearly coccoid, Gram-negative rods; also small Gram-positive rods resembling corynebacteria, and big Gram-negative cocci. A few small, short, irregular, acid-fast rods were found. Some extensive vegetative actinomyces-mycelia were present, although not abundantly, largely Gram-negative; chains of actinomyces-spores were seen here and there. A fair amount of fungus mycelium was seen, especially after 7 days; later, most of the hyphae appeared badly staining and decayed, often surrounded by Gram-negative bacteria which sometimes formed actual sheaths around them. Some fungal spores, apparently *Fusarium*, were seen after 30 days, and a few nematode-larvae were observed in this, as well as in the next soil.

b. Soil + Cellulose.—The bacterial flora did not undergo any considerable change, compared with the control soil; some beautiful pictures of Gram-positive, corynebacterium-like rods were seen (Pl. vi, fig. 12). Also the actinomycetes were little influenced; their mycelia were sometimes seen to form distinct spirals. The

TABLE 3.

Numbers of Bacteria, Actinomycetes and Fungi in Acid Grass Soil.

I. Soil with Addition of Cellulose.

Time.	Control Soil.				Soil+Cellulose.			
	Bact.	Act.	Fungi.		Bact.	Act.	Fungi.	
			1,000 per gm.	Spores %.			1,000 per gm.	Spores. %.
Start ..	11·7	6·5	603	40·5	11·7	6·5	603	40·5
7 days ..	47·4	4·2	759	26·6	53·2	4·4	1462	37·5
15 „ ..	30·5	5·7	597	40·5	38·2	4·0	1473	43·8
22 „ ..	—	—	454	40·1	—	—	999	55·1
30 „ ..	19·1	6·0	523	56·4	36·3	4·1	1217	(lost)

II. Soil with Addition of Mannite and Milk, after 3 days.

	Bacteria.	Actinomycetes.	Fungi.
Control Soil	25·8	3·5	606
Soil + Mannite	154·8	4·0	447
Soil + Milk	173·4	2·6	1440

fungi were entirely different: after 7 days the slides showed an abundant growth of fungus mycelia of mycomycetous type, with distinctly septate hyphae (Pl. vi, fig. 1). After 15–30 days the mycelia became much less abundant, largely Gram-negative and decaying, but fungal spores, which were few after 7 days, became quite numerous from the 15th day. They were partly spherical, measuring 2·0–2·5μ, resembling those of the ordinary Penicillia and Trichodermae, partly lemon-shaped, in fine long chains (Pl. vi, fig. 2). A pink *Penicillium* (*lilacinum*?) with spores of this characteristic shape appeared on the agar plates. These results are in good agreement with the plate counts, which show no very considerable change in the numbers of bacteria and actinomycetes as compared with the control soil, but by the 7th day a distinct increase in the numbers of fungi (chiefly *Trichoderma* and *Penicillium*), which remain quite high, owing to increasing spore production, as shown by both the microscopic and the plate method. The increase in fungi on the agar plates by the 7th day is somewhat less striking than one would expect after having seen the abundance of mycelium revealed by the microscopic method. The reason is probably that the mycelia at this period, being young and virile, are not easily broken up by the preparation of the soil suspension, so that they give rise to a comparatively small number of colonies, whereas the chains of spores, produced at a later period, are easily scattered.

In the second series of experiments, the control soil appeared microscopically quite like the previous.

b. Soil + Mannite.—This soil showed a considerable increase in bacteria (as also shown by the plate count), chiefly small Gram-positive rods, whereas the Gram-negative organisms did not increase much. *Azotobacter* was totally absent. Numerous small vegetative mycelia of actinomycetes were seen, but very few spores. Fungus mycelia were scarce; to judge by the plate count, the fungi seem actually depressed by the addition of mannite.

c. Soil + Milk.—A big increase in numbers of bacteria was shown both by the microscopic and the plate method. Nearly all bacteria on the slides were small or middle-sized, plump, Gram-negative rods. Vegetative actinomyces-mycelia were present. but largely Gram-negative; very few spores. On the agar plates the number of actinomycetes appeared definitely depressed. The slides showed a very strong development of fungus mycelium: mainly thick, non-septate, profusely branching hyphae of the *Mucor*-type. A corresponding increase in fungi was seen on the agar plates, chiefly represented by Mucoraceae.

The plate counts from the soils without additions are reproduced in Table 4. These soils were samples that had previously been used for microbiological analyses (Jensen, 1934); their character has been described under the corresponding numbers in Table 1 in the paper referred to. The air-dried samples (with the exception of No. 23, which was a freshly taken sample) were remoistened to 60–70% of their water-holding capacity and incubated for 7–14 days at room temperature.

TABLE 4.

Numbers of Microorganisms in Soils without Additions.

Soil No.	Incubation.	Bacteria.		Actinomycetes. Mill. per gm.	Fungi.	
		Mill. per gm.	Corynebact. %.		1,000 per gm.	Spores %.
15	10 days	(none)	—	(none)	373	53
25	14 ,,	1·4	18	1·3	90	—
6	10 ,,	3·5	22	0·2	17	—
16	14 ,,	10·2	4	0·2	359	84
4	12 ,,	23·0	38	1·1	13	—
9	14 ,,	24·4	46	0·9	43	—
3	7 ,,	47·8	50	11·2	425	37
23	12 ,,	58·8	41	11·1	498	22

Microscopical examination gave the following results:

No. 15.—Extremely poor in bacteria, nearly all Gram-negative rods, some of them quite big, club-shaped, containing spores, apparently clostridia; some free spores are also seen. A few Gram-negative actinomyces-mycelia are seen, but no actinomyces-spores. Bacteria and actinomycetes failed altogether to develop on agar. There was a fair amount of fungal hyphae, but mainly Gram-negative and decayed-looking, and a considerable amount of fungal spores, some lemon-shaped, others spherical, of *Penicillium*-type. In agreement herewith, the flora on agar was found to consist almost entirely of Penicillia, and a large fraction was represented by spores.

No. 25.—Poor in bacteria, although richer than the previous. Mainly small or middle-sized Gram-negative rods, some of the latter in long chains. A few small Gram-positive rods and big Gram-negative cocci. Fair amount of vegetative actinomyces-mycelia, partly Gram-negative, but very few spores. Large amount of fungal hyphae, partially Gram-negative and surrounded by bacteria; a few spores of *Penicillium*-type.

No. 6.—No mycelia of fungi or actinomycetes could be detected. The examination for bacteria was difficult owing to the fact that the colloids of this soil retained the Gram-stain quite tenaciously, but no large number of bacteria appeared to be present.

No. 16.—Very poor in bacteria, practically only small Gram-negative rods, some of them in chains and filaments. Fair amount of vegetative actinomyces-mycelia, but no spores. Fungal hyphae scant, Gram-negative or nearly unstained.

No. 4.—Poor in bacteria, almost exclusively plump, medium-sized, Gram-negative rods, mainly in big irregular colonies. No fungi or actinomycetes could be detected at all.

No. 9.—Rather poor in bacteria; mainly small Gram-negative rods in irregular colonies, also a number of small Gram-positive rods in pairs or small clusters. Some fungus mycelia are present, but no fungal spores or actinomyces-mycelia, and only few actinomyces-spores.

No. 3.—Rather poor in bacteria, which mostly occur in regions with much colloidal material. Small short Gram-positive rods and cocci are most common, whereas Gram-negative organisms are rare. Vegetative mycelia of actinomycetes are not visible, but some spores are seen. Fair amount of fungus mycelium. It is to be noted that this soil, owing to its high humus content, was of a very coarse-crumbly structure, so that a close contact with the slide may not have been established.

No. 23.—Rich in bacteria, still more so than the control soils A and B of the previous series. The majority of the bacteria are small, slender, Gram-negative rods, mostly in colonies of varying size; some plump Gram-negative rods were also common. Small Gram-positive rods, big Gram-negative cocci and long Gram-negative filaments are present in smaller numbers. Some of the Gram-negative rods show the same angular arrangement as the corynebacteria. Vegetative actinomyces-mycelia (largely Gram-negative) and actinomyces-spores are found, but not very frequently. Large amount of fungus mycelium, but the hyphae are mostly Gram-negative, often surrounded by clumps of bacteria. Very few fungal spores are seen.

There is thus in this series a rough parallelism between the apparent density of bacteria on the slides and their numbers in the plate counts, and the percentages of fungal spores as shown by the plate method coincide to some extent with the amount of mycelium on the slides, soil No. 16 with its high proportion of spores being very poor in mycelia, and No. 23 with its low spore-percentage being rich herein.

Discussion.

The decomposition experiments just described have shown that changes in the soil microflora, quantitative as well as qualitative, are revealed both by the Rossi-Cholodny method and the plate method, the results of which are largely in agreement with each other. So far, the results of the microscopic method might

also have been obtained by a judicious application of the plate and dilution methods, which, moreover, have the advantages of leading to quantitative results and of showing the state of the microflora (or, more correctly, a part of it) at a definite moment. The weakness of the microscopic method (apart from the facts that it does not give cultures of the organisms observed, and that most organisms cannot be identified simply by their morphology) is, firstly that we cannot tell whether the picture seen on the slides has been produced soon after the slide was placed in the soil, or just before it was removed (this is especially of importance when the slides have been left in the soil for several weeks), and secondly, that the distribution of the organisms on the slides is always too irregular to admit of a reliable counting. On the other side it has its advantages where the plate method falls short. It is able to inform us whether organisms not amenable to the study by cultural methods are active in the soil, and it shows the configuration of bacteria in colonies and their location around organic matter represented, for instance, by decaying fungal hyphae. If organisms of a characteristic morphology appear in great abundance on agar plates, it may also show whether or not these organisms make out a significant part of the total soil flora (cf. *Azotobacter* in soil plus mannite). It is also interesting to note that the microscopic method shows the bulk of the soil bacteria usually to be represented by small rods, as also found by Conn (1918), Kühlmorgen-Hille (1928), Cholodny (1930), and Demeter and Mossel (1933). The cocci, which Winogradsky (1925) and Koffmann (1931) found most numerous, are generally present, but in much smaller numbers. The majority of the bacteria are seen in nearly all cases to be Gram-negative, but Gram-positive rods, which seem to be corynebacteria, are also represented. Their relative abundance seems much smaller than shown by the plate method (cf. Table 4, and a previous paper—Jensen, 1933); it is to be remembered, however, that a certain proportion of the Gram-negative bacteria are doubtless cells that have been dead for a long time, among which there may be numerous corynebacteria no longer stainable by the Gram method. The microscopic method gives instructive pictures of the actinomycetes, showing their presence both as mycelium and as spores, which cannot as yet be distinguished from each other by any cultural method. It is noteworthy that the chains of actinomyces-spores are almost entirely confined to those parts of the slides that are free from soil colloids—evidently because the formation of the spores takes place in the air-filled pores of the soil (cf. Kubiena, 1932). The sporulation is seen sometimes to occur very rapidly, as in soil A with dry milk. These results sound a note of warning against an uncritical interpretation of microscopic counts of bacteria in soils where a rich sporulation of actinomycetes takes place; the spore-chains are very easily broken up and scattered when a soil suspension is prepared, in which case the spores will almost inevitably be counted as bacteria (cf. the remarks of Koffmann, 1931), since it is almost impossible to distinguish microscopically between bacteria and detached actinomyces-spores. There is also a possibility that the microscopic method may not always give an unbiassed picture of the actinomyces-flora: it is conceivable that where air-spaces are in contact with the slide, aerial hyphae of actinomycetes may grow out from the soil and adhere to the glass, where they appear as chains of spores, while the vegetative mycelia, from which these hyphae are produced, may remain in the soil. The growth of fungi in the soil, too, is finely demonstrated by the microscopic method, as also pointed out by Cholodny

(1930) and Conn (1932), and it seems to show the presence of young vegetative mycelia rather better than the plate method. Both methods agree in demonstrating the existence of fungal spores, sometimes in large numbers, in soils both with and without additions of organic matter; it seems, therefore, that the conclusion of McLennan (1928), according to whom the fungi exist almost entirely as mycelia in the soil, cannot be valid in general, at least as far as soils incubated in the laboratory are concerned. Finally, it is noteworthy that the existence of free-living amoebae in the soil can also be demonstrated by the microscopic method. The forms seen here (especially in soil A with milk and keratin) were quite large organisms which Koffmann (1931) considers aquatic forms, and therefore only active in soil under conditions of excessive moisture (soil suspensions which are allowed to stand). The present results seem to suggest that they may also become active under certain other circumstances.

The general impression is thus, that the Rossi-Cholodny method and plate method (supplemented by the dilution method, if desired) compensate each other's disadvantages to a large degree, and when combined they may yield very valuable information on the changes in the micropopulation of the soil in decomposition experiments with organic matter. Direct counts of microorganisms, especially by the method of Gray and Thornton (1928), may of course be used together with them, and may prove a still further aid in such studies.

Summary.

The Rossi-Cholodny method for direct microscopical examination of the soil micropopulation was compared with the plate method in decomposition experiments with organic compounds in soils of different character.

The results of the two methods were found generally to agree with each other, and since they largely compensate each other's disadvantages and serve as a control upon each other, their combined use seems very much to be recommended for the study of the soil microflora.

A differentiation between Gram-positive and Gram-negative soil organisms may be obtained by staining according to the Gram method and using Rose bengale or erythrosine as a counterstain.

The method may also be adapted to the study of acid-fast microorganisms in the soil.

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EXPLANATION OF PLATE VI.

Staining with Gram plus Rose bengale, magnification × 750, unless otherwise stated. 1.—Fungal mycelium. Soil B + cellulose, 7 d. 2.—Fungal spores. Same, 15 d. 3.—*Actinomyces*-mycelium. Same, 7 d. 4.—*Actinomyces*-mycelium. Soil A + keratin, 7 d. 5.—*Actinomyces*-spores. Soil A + milk, 3 d. 6.—*Actinomyces*-spores. Soil A + keratin, 3 d. 7.—Amoeba containing actinomyces-spores. Soil A + milk, 11 d. 8.—*Azotobacter*. Soil A + mannite, 11 d. 9.—Gram-negative filaments, forming "spools". Soil A + cellulose, 10 d. 10-12.—Gram-positive rods (corynebacteria?). 10: Soil A + keratin, 7 d. 11: Soil A + mannite, 16 d. 12: Soil B + cellulose. 13.—Acid-fast rods, stained with carbol-fuchsin, decolourized with 5% sulphuric acid. Soil A + paraffine, 10 d. Nos. 3, 4, 6, 7, 11, 12 and 13 have been retouched in order to make the organisms clearly visible among the soil colloids.